Exhibit D

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The Presence of Fatty Acids in Human α-Fetoprotein*

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DAVID C. PARMELEE, MERLE A. EVENSON, AND HAROLD F. DEUTSCH

From the Departments of Physiological Chemistry and Medicine, University of Wisconsin Medical Center, Madison, Wisconsin 53706

α-Fetoprotein has been prepared from human fetal tissue by procedures utilizing DEAE-Sephadex, concanavalin A-Sepharose, and isoelectric focusing. A major and a minor component with isoelectric points of 4.7 and 5.3, respectively, have been isolated and are similar to those prepared under various conditions by other investigators. The 4.7 material contains 2.4 mol of fatty acids/mol of protein, whereas the minor component is fat-free. The relative amounts of fatty acid vary somewhat with different preparations. The ranges found in three isolates were as follows: palmitic acid (8 to 11%), stearic acid (2 to 5%), oleic acid (10 to 28%), linoleic acid (7 to 15%), arachidonic acid (12 to 39%), and 4,7,10,13,16,19-docosahexaenoic acid (16 to 42%). Human fetal serum albumin contained 0.7 mol of fatty acid/mol of protein, with arachidonic acid and the docosahexaenoic acid comprising only 11.4% of the total. Removal of fatty acids by treatment with charcoal converted α-fetoprotein into material with an isoelectric point of pH 5.3. Addition of arachidonic acid to the lipid-free protein restored it to protein with a pH 4.7 isoelectric point, typical of the major native component. The possible role of the fatty acids in α-fetoprotein on the inhibition of various lymphocyte functions is projected.

Human α-fetoprotein is a protein found in relatively high concentrations in the serum of fetal animals. It has attracted considerable attention due to its reappearance in several pathological conditions in adults. Abelev and co-workers (1) discovered that in many cases of hepatocellular carcinoma, the serum concentrations of α-fetoprotein were equal to fetal levels. This observation led to the classification of the protein as a tumor-specific embryonic antigen until more sensitive techniques demonstrated its increased serum concentration in tetratocarcinomas and some nonmalignant diseases (2-4). However, the protein remains an important clinical entity since the extremely high levels are unique to hepatocellular carcinoma.

The biological function of α-fetoprotein is not known, although various experimental observations have directed attention toward several possible roles. The specific binding of estradiol to the protein indicates that it may be important in the transport of this steroid (5) and it has been suggested to possibly play a role in protecting the embryo against the toxic effects of the high estrogen levels of the mother (6). More recently, a role of immunosuppression had been postulated by Murgita and Tomasi (7), who demonstrated that it suppresses both the primary and secondary *in vitro* antibody responses of lymphocytes. They also observed that α-fetoprotein can suppress allogenic and mitogen-induced lymphocyte transformation in mice (8). However, other workers have not found consistent effects of the protein on these immune responses and transformations (9, 10). The immunofluorescence studies of Dattwyler *et al.* (11) suggest that α-fetoprotein receptors are on the surface of certain T cell lymphocyte populations in mice. These findings, in conjunction with the abortogenic activity of antiserum to α-fetoprotein (12), indicate that it may be involved in preventing an immune response against fetal tissues or tumors. The similarities of α-fetoprotein and serum albumin as evidenced by sequence homologies (13) and immunological cross-reactivities (14) have suggested α-fetoprotein may serve as a fetal form of albumin by functioning to transport important biological molecules and to maintain osmotic equilibrium (15, 16).

The drastic conditions often employed in isolating α-fetoprotein may result in modification of its biological activity. For this reason, we have developed a relatively mild procedure for purifying α-fetoprotein from fetal tissue. The protein prepared by this method has been found to contain a variety of fatty acids, some of a type not noted in adult albumin or seen only in trace amounts. The fatty acids can be removed from the protein by using procedures analogous to those employed to remove fatty acids from albumin (17). This protein can be reconstituted with fatty acids to yield material with properties similar to the original preparation. The possible importance of the fatty acid components of α-fetoprotein in mediating the inhibition of lymphocyte functions is projected.

MATERIALS AND METHODS

Preparation of Con A[1]-Sepharose

Con A from 500 g of jack bean meal (Sigma) was purified on a column (3.5 × 90 cm) of Sephadex G-100 (Pharmacia) by the method of Agrawal and Goldstein (18). It was then coupled to 250 ml of Sepharose 4B (Pharmacia) by the cyanogen bromide procedure (19). A column of the resulting complex was equilibrated with pH 6.0, 0.1 M sodium acetate containing 1.0 M NaCl, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$.

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[1] The abbreviations used are: Con A, concanavalin A; pI, isoelectric point; 16:0, palmitic acid; 18:0, stearic acid; 18.1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, 4,7,10,13,16,19-docosahexaenoic acid; 24:1, nervonic acid.

Purification of α-Fetoprotein

Preparation of Fetal Extract—The procedures described in the purification are based on 100 g of fetal tissue. Four- to five-month-old fetuses obtained immediately after therapeutic abortions served as starting material. Homogenates were prepared by blending fetal tissue with one-third their weight of 0.15 M NaCl in a Waring Blendor. The homogenate was clarified by centrifugation at 4400 × *g* for 5 min and the resulting residue was washed twice with the above weight of 0.15 M NaCl. The supernatants were combined to yield an extract of about 125 ml/100 g of starting tissue. The aggregated lipid material which formed during centrifugation was removed by filtration through glass wool. The fetal extract was then dialyzed against pH 6.2, 0.04 M potassium phosphate at 4°.

Chromatography on DEAE-Sephadex—The suspended material in the dialyzed extract was removed by centrifugation at 19,600 × *g* for 20 min. One liter of packed DEAE-Sephadex A-50 (Pharmacia) equilibrated with the dialysis buffer was then added to the resultant supernatant. After slow stirring at 4° for 6 h, the DEAE-Sephadex slurry was packed into a column (5.1 × 75 cm) and washed with 1 liter of the buffer. The adsorbed proteins, which included most of the α-fetoprotein, were eluted with the above phosphate buffer containing 0.5 M NaCl.

The protein fractions were pooled, dialyzed against the pH 6.2, 0.04 M potassium phosphate buffer, and concentrated to a volume of 10 ml in a Diaflo apparatus using a UM-10 membrane. The sample was then adsorbed on a column of DEAE-Sephadex equilibrated with the pH 6.2, 0.04 M potassium phosphate. Elution was effected by means of a linear gradient using 3 liters of pH 6.2, 0.04 M potassium phosphate buffer containing 0.15 M NaCl and an equal volume of this buffer containing 0.32 M NaCl. The presence of α-fetoprotein in the various fractions was determined by the use of a monospecific antibody[2] and fractions were pooled as indicated in Fig. 1.

Chromatography on Con A-Sepharose—α-Fetoprotein is difficult to separate from albumin due to similarity of molecular weight and isoelectric point. The absence of carbohydrate in serum albumin, however, permits removal of the α-fetoprotein by affinity chromatography on Con A-Sepharose (20). The fractions containing α-fetoprotein which eluted from the DEAE-Sephadex column (as shown in Fig. 1) were concentrated to a volume of 10 ml in the Diaflow apparatus and then dialyzed against pH 6.0, 0.1 M sodium acetate containing 1.0 M NaCl, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$. The protein solution was then applied to a column of Con A-Sepharose equilibrated with this buffer, washed with approximately 0.7 column volumes of it, and then eluted with the same buffer containing 1% α-methylglucoside. The result shown in Fig. 2 was obtained. The first component which reacted only with the antibody to albumin eluted within 1 column volume after the sample application. Its elution was not dependent on the addition of the buffered 1% α-methylglucoside, whereas the second component eluted only after 1 column volume of the latter buffer. This component reacted only with specific antibody to α-fetoprotein.

Isoelectric Focusing—The fractions containing α-fetoprotein were pooled, exhaustively dialyzed against distilled water, and subjected to isoelectric focusing. As previously noted by other authors (16), two components, both reacting with antibody to α-fetoprotein were resolved (see Fig. 3). These fractions were pooled as indicated, dialyzed against distilled water, and stored at −20°.

Preparation of Antiserum—The major component of the highly purified preparation of α-fetoprotein was then used to prepare additional rabbit antiserum. The protein in complete Freund's adjuvant was administered to rabbits, each animal being given a total of 1 mg distributed equally in each of the hind leg foot pads as well as in two subcutaneous sites. Each week an additional 0.5 mg was given subcutaneously. After 4 weeks, the animals exhibited a serum antibody titer capable of detecting 10 μg of α-fetoprotein/ml by the interfacial ring test (21). The rabbits were then bled weekly by cardiac puncture and subcutaneous injections of 0.5 mg of α-fetoprotein in complete Freund's adjuvant were given concurrently to maintain the titer. Antiserum to serum albumin was prepared in a similar manner. These antibody preparations were employed to detect the presence of α-fetoprotein and albumin in various protein fractions and to ensure the purity of the α-fetoprotein preparations employed in various studies.

[2] This antiserum was a gift from Professor H. Hirai.

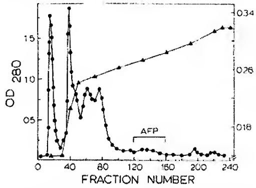


FIG. 1. Chromatogram illustrating the fractionation of a fetal extract from 475 g of tissue on a column (5.1 × 52 cm) of DEAE-Sephadex A-50 equilibrated with pH 6.2, 0.04 M potassium phosphate. The proteins were eluted at 4° by means of a linear gradient established by the addition of 3 liters of the buffer containing 0.32 M NaCl to 3 liters of one containing 0.14 M NaCl (▲——▲, molarity of added NaCl; ●——●, optical density at 280 nm). *AFP*, α-fetoprotein.

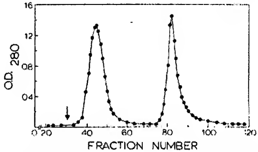


FIG. 2. Affinity chromatography on Con A-Sepharose of the α-fetoprotein fraction from 475 g of fetal tissue separated by DEAE-Sephadex. A column (2.5 × 41 cm) was equilibrated with pH 6.0, 0.1 M sodium acetate containing 1.0 M NaCl, 0.001 M $MnCl_2$, 0.001 M $CaCl_2$. The α-fetoprotein was eluted by the application of this acetate buffer containing 1% α-methylglucoside at the point indicated by the *arrow*. Fractions of 4 ml were collected using a flow rate of 6 ml/h/cm² (●——●, optical density at 280 nm).

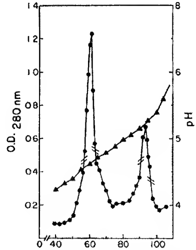


FIG. 3. Result for an isoelectric focusing experiment on the α-fetoprotein fraction (equivalent to 475 g of fetal tissue) separated by affinity chromatography on Con A-Sepharose. A pH 4 to 6 Ampholine was employed and fractions of 1 ml were collected (▲——▲, pH; ●——●, optical density at 280 nm). Fractions pooled to provide the pI, 4.7 and 5.3 materials are indicated by the *hash marks* on the elution profiles.

Quantitation of Fatty Acids

Extraction – Six milliliters of a solvent mixture consisting of 40 volumes of isopropyl alcohol, 10 volumes of *n*-heptane, and 1 volume of 1 N sulfuric acid (22) was added to 1.0 ml of α-fetoprotein solution containing from 0.5 to 3.5 mg of protein, to which 2 to 5 μg of *n*-heptadecanoic acid had been added as an internal standard. The mixture was mixed for 30 s in screw-capped test tubes (15 × 125 mm) to effect extraction of fatty acids. Two milliliters of distilled water was then added and the sample mixed for 15 s. Three milliliters of *n*-heptane was added and this solution again mixed for 15 s. A 3.5-ml aliquot of the upper organic phase was then removed, placed in the same type tube, and the solvent removed at 35° with a stream of nitrogen.

Methylation – The dried lipid extract was refluxed for 3 min with 1 ml of BF_3-MeOH (14% w/v)/BCl_3-MeOH (10% w/v) (Applied Science Labs., Inc.). One milliliter of *n*-heptane and 1 ml of distilled water were then added and mixed for 15 s. A 0.85-ml aliquot of the upper phase was transferred to a conical test tube (10 × 75 mm) and taken to dryness at 35° with a stream of nitrogen.

Gas-Liquid Chromatography – The esterified sample was mixed with 50 μl of *n*-heptane to effect solution. An aliquot of it was analyzed with a model 402 Hewlett Packard High Efficiency Gas Chromatograph with dual hydrogen flame detectors using glass columns containing various packings (Applied Science Labs., Inc.). Isothermal conditions were maintained and gas flow rates of 25, 35, and 200 ml/min were employed for helium, hydrogen, and air, respectively.

Quantitation – The type and levels of methyl esters prepared from the α-fetoprotein extract were determined by comparing the heights of each component eluting with those of a mixture of standard fatty acids (Applied Science Labs., Inc., and Supelco, Inc.). Differences in extraction and methylation efficiencies of individual fatty acids were corrected for by the recoveries of the *n*-heptadecanoic acid employed as the internal standard. Estimates of precision were obtained by use of a stock solution of albumin to which arachidonic and docosahexaenoic acids had been added. The standard deviation was then calculated from the results of 13 separate experiments. The mean and standard deviations experienced for each of the fatty acids in the stock solution were as follows: 16:0 (1.11 ± 0.08), 18:0 (0.99 ± 0.05), 18:1 (0.74 ± 0.04), 18:2 (0.38 ± 0.02), 20:4 (1.48 ± 0.13), 22:6 (0.95 ± 0.05).

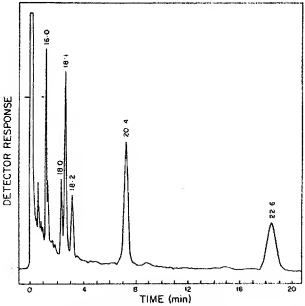
Defatting and Reconstitution of α-Fetoprotein

Preparation of Fatty Acid-free α-Fetoprotein – A modification of the method used by Chen (17) with albumin was employed. Forty-five milligrams of activated charcoal was added to a 15-mg sample of protein in 10 ml of distilled water at 0°. The pH was then carefully adjusted to 3.0 with 0.1 N HCl and the mixture incubated with shaking at 0° for 2 h. The solution was then centrifuged at 25,000 × *g* for 30 min at this temperature. The supernatant containing the lipid-free protein was then decanted from the charcoal and adjusted to pH 7.0 with 0.1 N NaOH.

Addition of Arachidonic Acid to Lipid-free α-Fetoprotein – An amount of the fatty acid sufficient to provide a 3- to 40-fold molar excess over the amount of protein to be employed was dissolved in *n*-heptane and added to a test tube (15 × 125 mm). The solvent was then removed at 35° with a stream of nitrogen. A solution of the lipid-free α-fetoprotein in distilled water was added and the contents allowed to react for 1 h at 22° with gentle shaking.

RESULTS

Identification of Fatty Acids Extracted from α-Fetoprotein – A chromatogram of the methyl esters of the fatty acid fraction which was extracted from α-fetoprotein is shown in Fig. 4. The first five components eluting were identified by comparing their relative retention times with those of a mixture of methyl esters of known fatty acids which eluted as shown in Fig. 4. The identifications of the slower eluting components designated as 20:4 and 22:6 were less certain due to the increased possibility that other fatty acid methyl esters might have identical relative retention times. Since the 20:4 component appeared to be arachidonic acid, preliminary efforts were directed to the identification of the 22:6 material, which at this stage of the experiments was designated as Component X. Preliminary experiments indicated that it was not a bile acid, cholesterol, glyceride, prostaglandin-type material, or estradiol. The latter material was investigated due to the known binding of this substance by α-fetoprotein. Sufficient Component X was isolated by gas chromatography for additional studies. Analysis of ozonolysis products gave no identifiable components. Mass spectrometric analysis was also employed, but no high molecular weight ion species was obtained that proved useful in identification. However, fragmentation ions



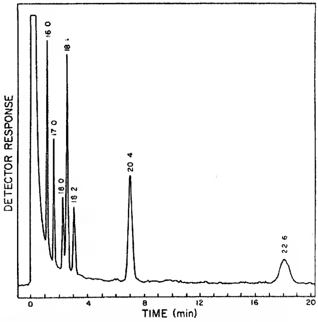


FIG. 4. Chromatogram illustrating the separation of the methyl esters of fatty acids by gas-liquid chromatography on a glass column (0.2 × 175 cm) packed with 3% Silar 5 CP on Gas-chrom Q, 80 to 100 mesh, at a temperature of 185°. The chain length and number of double bonds in each fatty acid are indicated by the numerals located over each peak. *Left*, fatty acid methyl esters of α-fetoprotein; *right*, standard fatty acid methyl esters.

similar to those seen with samples of methyl arachidonate were noted and suggested that a higher molecular weight unsaturated fatty acid was involved. Chromatography of a series of C-20 and C-22 fatty acids containing various degrees of unsaturation indicated that the compound in question was 4,7,10,13,16,19-docosahexaenoic acid. However, nervonic acid (24:1) was also found to have an identical relative retention time using the conditions shown in Fig. 4. When columns containing 3% OV-1 or 3% OV-225 were employed, the methyl ester of 24:1 was completely resolved from the methyl esters of 20:4 and 22:6. Furthermore when known amounts of the methyl esters of 20:4 and 22:6 were added to the fatty acid methyl esters obtained from α-fetoprotein and then chromatographed on the above types of column packings, each at several temperatures, chromatograms with symmetrical peaks of increased heights for both these components were obtained. Chromatograms illustrating these experiments are shown in Fig. 5. Similar results were obtained at a temperature at 195° and when a 3% OV-225 column at 200° and at 230° was employed.

Quantitation of Fatty Acids in α-Fetoprotein – The molar ratios of each fatty acid in the pH 4.7 isoelectric form of α-fetoprotein from three different preparations is shown in Table I. The values range from 2.39 to 3.09 mol of fatty acid/mol of protein and the unsaturated fatty acids account for 86 to 89% of the total. The quantities of the 20:4 and 22:6 components range from 54 to 70% of the total fatty acids and were found to show considerable variation in different preparations. Fetal albumin isolated from the same extract was found to contain 0.7 mol of fatty acid/mol of protein. The unsaturated fatty acids in this material are present in relatively high concentrations, but the 20:4 and 22:6 components comprise only 11% of the total. The fatty acid levels of adult albumin are also shown in Table I. The molar ratio of fatty acids per mol of albumin (2.44) is similar to that of α-fetoprotein, but only 2% of the total is due to 20:4. No 22:6 component was found. The unsaturated fatty acids bound to adult albumin consist mostly of 18:1 and account for only 32% of the total. The pH 5.3 isoelectric point fraction of α-fetoprotein was found to contain less than 0.01 mol of fatty acid/mol of protein.

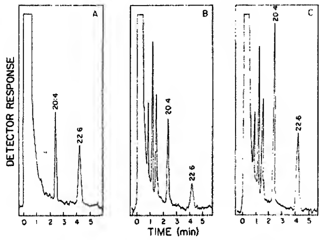


FIG. 5. Chromatograms illustrating the identification of the methyl esters of the 20:4 and 22:6 components of α-fetoprotein. The elution profile of a mixture of the methyl esters of arachidonic acid (20:4) and 4,7,10,13,16,19-docosahexaenoic acid (22:6) is shown in *A*, the methyl esters derived from α-fetoprotein in *B*, and a mixture of the two standards with the esters derived from α-fetoprotein in *C*. The chromatography was performed on a glass column (0.2 × 175 cm) packed with 3% OV-1 on Gas-Chrom Q, 100 to 120 mesh, at a temperature of 225°.

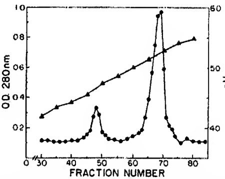


FIG. 6. Result for an isoelectric focusing experiment on 15 mg of the major pI 4.7 component of purified α-fetoprotein following its treatment with charcoal to remove fatty acids. A pH 4 to 6 Ampholine was employed and fractions of 1 ml were collected (▲—▲, pH; ●—●, optical density at 280 nm).

TABLE I

Concentrations of fatty acids extracted from α-fetoprotein and human serum albumin

Protein[a]	Moles fatty acid/mol protein[b]							Per cent of total fatty acids	
	16:0	18:0	18:1	18:2	20:4	22:6	Total	20:4 + 22:6	All unsaturated
AFP (pI 4.7 of Fig. 3)	0.21 (8)	0.05 (2)	0.66 (28)	0.17 (7)	0.29 (12)	1.01 (42)	2.39 ± 0.07	54	89
AFP[c] (pI 4.7)	0.20 (8)	0.14 (5)	0.28 (10)	0.17 (7)	0.99 (37)	0.88 (33)	2.66 ± 0.10	70	87
AFP[c] (pI 4.7)	0.33 (11)	0.11 (3)	0.51 (16)	0.46 (15)	1.20 (39)	0.49 (16)	3.09 ± 0.11	55	86
HSA[d] (fetal, pI 4.8)	0.10 (15)	0.02 (3)	0.37 (55)	0.10 (15)	0.05 (7)	0.03 (4)	0.70 ± 0.02	11	81
HSA[e] (adult, pI 4.8)	0.83 (34)	0.83 (34)	0.48 (20)	0.24 (10)	0.05 (2)	(0.00) (0)	2.44 ± 0.08	2	32

[a] The data in parentheses in this column refer to the source of protein and their isoelectric points.

[b] Based on molecular weights for human serum albumin (HSA) and α-fetoprotein (AFP) of 68,000 and 70,000, respectively. The fatty acid designations are referent to numbers of carbon atoms and double bonds (23). The values in parentheses are the percentages of the total fatty acids. The fatty acids were extracted from the various protein preparations and quantitated as described under "Materials and Methods." All determinations were done in triplicate unless otherwise stated.

[c] Lack of material permitted only a single fatty acid determination for these preparations.

[d] Purified by isoelectric focusing of first component eluting from the Con A-Sepharose affinity chromatography experiment shown in Fig. 2.

[e] This material was purified from serum by chromatography, salt fractionation, and isoelectric focusing techniques.

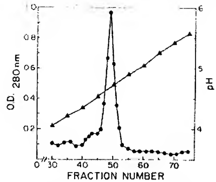


FIG. 7. Result for an isoelectric focusing experiment on 8 mg of lipid-free α-fetoprotein which was reconstituted with arachidonic acid. A pH 4 to 6 Ampholine was employed and fractions of 1 ml were collected (▲——▲, pH; ●——●, optical density at 280 nm).

Removal of Fatty Acids and Reconstitution – The result of isoelectric focusing a sample of α-fetoprotein from which fatty acids were removed by charcoal treatment is shown in Fig. 6. Approximately 84% of the material in the column is the lipid-free component with an isoelectric point of 5.3. More of the residual pI 4.7 material could be converted to the fat-free form by using higher ratios of charcoal to protein, or by longer periods of incubation at pH 3.0, or both. However, these conditions appear to result in decreased yields of total protein. Arachidonic acid was added to the lipid-free fraction in a molar ratio of 40:1 and then subjected to isoelectric focusing. The result shown in Fig. 7 indicates that all of the protein was converted to material with an isoelectric point of 4.7 characteristic of the major component isolated from fetal tissues. Limitation of material to the present time have prevented more extensive investigations of this type.

DISCUSSION

Human α-fetoprotein, separated by the relatively mild method described, binds all of the fatty acids found in the serum albumin of adults and in addition, contains 4,7,10,13,16,19-docosahexaenoic acid (22:6) which is not a normal constituent of the latter protein. The relative proportions of C-16 and C-18 fatty acids in α-fetoprotein agree well with that of human serum albumin as reported by Saifer and Goldman (23), but differ somewhat from those of the human serum albumin analyzed in this work. This discrepancy may reflect differences in purification procedures. Human fetal serum albumin had only about one-third the amount of fatty acids as α-fetoprotein and also has significantly lower amounts of the 20:4 and 22:6 components. The concentration of albumin is from 5 to 7 times greater than α-fetoprotein in fetuses of the age employed in this study (24) which suggests that the latter protein has not only a greater affinity for binding fatty acids in general, but also has a greater specificity for the unsaturated forms than does albumin.

The possibility exists that the 20:4 and 22:6 components of the α-fetoprotein could have been derived from fetal tissues as the result of the homogenization procedure. However, it would be anticipated that the serum albumin, a protein with a high affinity for fatty acids and present at much higher concentration than α-fetoprotein in the fetus, would also have shown the presence of the 22:6 component. The relatively high content of unsaturated fatty acids in α-fetoprotein, particularly of 20:4 and 22:6, may indicate that they serve some specific function in the fetus when combined with this protein.

Isoelectric focusing experiments demonstrated that α-fetoprotein shows considerable heterogeneity. Approximately 84% of it had an isoelectric point of 4.7 while the remaining 16% was isoelectric at 5.3. It is not known if this heterogeneity exists *in vivo* or if it is a consequence of the isolation procedure. The method we employed was relatively mild compared to those of some other workers who have utilized conditions entailing 3 M sodium thiocyanate (9), 8 M urea (25), 1 M acetic acid (26), 15% dioxane at room temperature (27), 4 M guanidine HCl (7), and pH values as low as 1.8 (28) in their isolation procedures. These conditions may modify the protein since the biological activity of mouse α-fetoprotein is altered by even the relatively mild procedure of dialysis against 0.5 M KCl (26). One of our concerns is that the variation in fatty acid contents that we have experienced with preparations of α-fetoprotein may reflect small loses that may be difficult to circumvent. Nevertheless, the question of the relationship of the degree of heterogeneity of α-fetoprotein to the isolation method can not be ascertained at the present time.

The two isoelectric components noted in our α-fetoprotein preparation have been previously observed and it has been suggested that they may reflect differences in sialic acid content (16). However, others have indicated the above forms contain the same amounts of this carbohydrate (29). The results described in the present work on the human protein strongly suggest that the difference between the isoelectric point 4.7 and 5.3 components is due to the presence and absence of fatty acids, respectively. The binding of fatty acids may be co-operative since we find no intermediate forms between the material that lacks fatty acids and the form with an isoelectric point of 4.7 which contains 2 to 3 mol of fatty acid/mol of protein.

Rat and mouse α-fetoprotein have been reported by some investigators to show microheterogeneity (30, 31). These workers, however, do not agree as to whether the variations in sialic acid content are responsible for these differences. It would be interesting to determine whether the various electrophoretic forms of these proteins also relate to variations in fatty acid content.

A considerable interest has recently developed relative to a possible biological role for α-fetoprotein in the mixed lymphocyte test and in the inhibition of lymphocyte transformations induced by mitogens and antiserum to human thymocytes. Some workers have reported that human α-fetoprotein, isolated by different procedures, suppresses human lymphocyte function (29, 32–34) while others indicate an augmentative effect (35). Much of the controversy may relate to the heterogeneity discussed above, since various forms of α-fetoprotein could have different effects. For example, the ability of human α-fetoprotein to suppress the mitogenic response of human lymphocytes has been correlated with the relative amount of the most acidic electrophoretic component present in the serum of patients with hepatocellular carcinoma (29, 34). These workers found that the presence of sialic acid was not necessary for the effects noted, but others have indicated that this component is required for the inhibition of antibody formation in mouse splenic lymphocyte cultures (36). Additional controversy results from α-fetoprotein inhibiting some *in vitro* immune phenomena in mice and rats, but not exhibiting general immunosuppressive functions *in vivo* (9). These types of conflicting results make an interpretation of the biological function of α-fetoprotein difficult. The relatively drastic conditions often employed in the purification of this protein could be related to the inconsistent results and be a

reflection of variations in its content of fatty acids.

Several observations may indicate that specific fatty acids are important in the biological activity of α-fetoprotein. It is interesting to note that this protein as well as serum albumin derived from human cord blood inhibits various lymphocyte functions, but that albumin of adults was without significant activity (37). The latter protein differs from the others in that it lacks the 22:6 component. Both the 20:4 and 22:6 fatty acids are present in fetal albumin and α-fetoprotein, although we have found that they are present in the latter protein in concentrations 6 and 35 times greater, respectively. Further evidence suggesting that fatty acids may have a functional role relates to the possibility that they increase the binding of estradiol by α-fetoprotein in a manner analogous to their influence on steroid binding by albumin (38). The presence of estrogen in mouse α-fetoprotein has been shown to be necessary for its functional activity in the inhibition of mitogen-induced transformation of lymphocytes (26). However, it is not known whether this relates to the amounts or types of fatty acids bound to the mouse α-fetoprotein. We are presently investigating the importance of fatty acid components of α-fetoprotein in the mixed lymphocyte reaction and on the inhibition of lymphocyte transformation by Con A and phytohemagglutinin. The ability to remove fatty acids from α-fetoprotein and to reconstitute the protein as shown in the present investigation will permit studies of the effects of individual fatty acids or combinations of them on such immunological processes.

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